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Antisense inhibition of NADH glutamate synthase impairs carbon/nitrogen assimilation in nodules of alfalfa (*Medicago sativa* L.)

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Summary

Legumes acquire significant amounts of nitrogen for growth from symbiotic nitrogen fixation. The glutamine synthetase (GS)/NADH-dependent glutamate synthase (NADH-GOGAT) cycle catalyzes initial nitrogen assimilation. This report describes the impact of specifically reducing nodule NADH-GOGAT activity on symbiotic performance of alfalfa (*Medicago sativa* L.). Four independent transgenic alfalfa lines, designated GA89, GA87, GA88, and GA82 (for *GOGAT* antisense), containing an antisense *NADH-GOGAT* cDNA fragment under the control of the soybean leghemoglobin (*lbc3*) promoter were evaluated. The GA plants were fertile and showed normal growth in non-symbiotic conditions. The *NADH-GOGAT* antisense transgene was heritable and the T_1 plants showed phenotypic alterations – similar to primary transformants. Clonally propagated plants were inoculated with *Sinorhizobium meliloti* after rooting and the symbiotic phenotype was analyzed 21 days post-inoculation. Nodules of each GA line had reduced NADH-GOGAT activity, ranging from 33 to 87% of control plants, that was accompanied by comparable decreases in RNA and protein. Plants from the GA89 line, with the lowest NADH-GOGAT activity (*c.* 30%), presented a strikingly altered symbiotic phenotype: concomitantly activities of key enzyme for carbon and nitrogen assimilation decreased; nodule amino acids and amides were reduced while sucrose accumulated. Antisense *GOGAT* plants were chlorotic, reduced in fresh weight, and had a lower N content than control plants. Photosynthesis was also impaired in antisense plants. Specifically, reducing NADH-GOGAT in nodules resulted in plants having impaired nitrogen assimilation and altered carbon/nitrogen metabolic flux.

Keywords: NADH-glutamate synthase, antisense transgenic alfalfa, *S. meliloti*–alfalfa symbiosis, C/N metabolism in nodules, symbiotic N fixation.

Introduction

Alfalfa (*Medicago sativa* L.) can acquire nitrogen for growth from the atmosphere, through symbiotic nitrogen fixation with the bacterium *Sinorhizobium meliloti.* In this association, bacteroids in root nodules reduce nitrogen to ammonia which is released into the cytosol of the host cell. Ammonia is assimilated to organic nitrogen by the cooperative activities of glutamine synthetase (GS, EC 6.3.1.2) and NADH-dependent glutamate synthase (NADH-GOGAT, EC 1.4.1.14) (Temple *et al.*, 1998b). GS catalyzes the incorporation of ammonia into glutamate, producing glutamine while NADH-GOGAT catalyzes the transfer of the amide group of glutamine to 2-oxoglutarate, resulting in the formation of two molecules of glutamate.

In plants, GOGAT occurs in two distinct forms that use NADH (NADH-GOGAT, EC 1.4.1.14) or ferredoxin (Fd-GOGAT, EC 1.4.7.1) as electron carriers. These enzymes differ in their location within the plant and are the products of distinct genes (Lam *et al.*, 1996; Temple *et al.*, 1998b). While Fd-GOGAT is more abundant in photosynthetic tissue, NADH-GOGAT is found primarily in non-green tissue such as roots and legume root nodules and is located in plastids (Lam *et al.*, 1996; Trepp *et al.*, 1999a). In nitrogen-fixing alfalfa nodules NADH-GOGAT activity has been

found to increase markedly during nodule development, and this activity is associated with a single form of the enzyme (Gregerson *et al.*, 1993; Temple *et al.*, 1998b). Trepp *et al.* (1999a) and Gregerson *et al.* (1993) demonstrated that NADH-GOGAT is encoded by a single gene in alfalfa. NADH-GOGAT activity is maintained at low or undetectable levels in other tissues of alfalfa (Vance *et al.*, 1995), but is detectable in flowers (Schoenbeck *et al.*, 2000).

Glutamate and glutamine serve as nitrogen donors for the biosynthesis of other nitrogen compounds that are transported from the nodule to other plant organs. The principal compounds transported from alfalfa nodules are amides such as asparagine and glutamine (Temple *et al.*, 1998b). Asparagine and aspartate are synthesized through the action of aspartate amino transferase (AAT, EC 2.6.1.1) and asparagine synthetase (AS, EC 6.3.5.4).

Ammonium assimilation in the nodule depends on carbon skeletons and energy. Sucrose derived from photosynthesis is the main carbon compound transported from shoots to nodules. In mature nodules, sucrose is primarily cleaved by sucrose synthase (SS, EC 2.4.1.13) to UDP-glucose and fructose. Gordon *et al.* (1997, 1999) have described the critical role of SS in the development and function of soybean nodules. Free hexoses produced in the nodule via SS are phosphorylated by hexokinases and are metabolized to phosphoenolpyruvate. Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) catalyzes the irreversible carboxylation of phosphoenolpyruvate to oxaloacetate. CO_2 fixation via PEPC provides a substantial amount of carbon skeletons for ammonium assimilation in the nodule; in alfalfa it provides up to 25% of the supplied carbon (Vance *et al.*, 1994).

The development of mutants or transgenic plants, low in a single enzyme activity, is useful in determining which enzyme(s) might catalyze the rate-limiting step in the overall process of nitrogen fixation and ammonium assimilation. *Arabidopsis*, barley, and pea mutants lacking Fd-GOGAT (Avila *et al.*, 1993; Lea and Forde, 1994; Oliveira *et al.*, 1997; Somerville and Ogren, 1980) as well as antisense Fd-GOGAT transgenic tobacco plants (Ferrario-Méry *et al.*, 2000) have been well characterized. These studies demonstrated the major role of Fd-GOGAT in the re-assimilation of ammonia from photorespiration, in photosynthetic tissues.

Owing to the tetraploid nature of alfalfa, it has not been possible to obtain mutants lacking ammonia assimilating enzymes. Nevertheless, antisense transgenic alfalfa plants impaired in a nodule enzyme activity, such as GS1 (Harrison *et al.*, 2000), PEPC (Schulze *et al.*, 1998), and AAT-2 (Mett *et al.*, 1996) have been reported. Antisense strategy has also been used to reduce urcase activity in *Vigna* (Lee *et al.*, 1993). Recently Schoenbeck *et al.* (2000) reported the characterization of a single transgenic alfalfa plant containing an antisense *NADH-GOGAT* under the control of the nodule-enhanced *AAT-2* promoter. NADH-GOGAT activity was reduced by some 40–50% in nodules, but there was a modest impact on the symbiotic phenotype. Moreover, antisense *NADH-GOGAT* expression was driven by the *AAT-2* promoter which is not specific for root nodules. Because the *AAT-2* promoter also resulted in antisense *NADH-GOGAT* expression in flowers pleiotropic effects on pollen development were observed.

In this work, we report the physiological characterization of four transgenic alfalfa (*M. sativa* L.) plants with the chimeric gene composed by the soybean nodule-specific leghemoglobin (Lb) gene promoter (Christensen *et al.*, 1989) fused in the antisense orientation of a cDNA fragment from alfalfa *NADH-GOGAT* (Gregerson *et al.*, 1993). Plants from each line showed a different percentage of remaining NADH-GOGAT activity in the nodules, ranging from 33 to 87%. The most deleterious symbiotic phenotype was observed in the plants with the lowest nodule NADH-GOGAT activity. These plants were drastically affected in the carbon/nitrogen assimilation in the nodule and in nodule anatomy, indicating the crucial role of this enzyme in symbiotic metabolism.

Results

Selection of transgenic alfalfa plants

Plasmid pMRGA (Figure 1a) contained the antisense construct of a cDNA fragment (5.3 kb) from nodule-enhanced alfalfa *NADH-GOGAT* (Gregerson *et al.*, 1993) fused to the 2-kb promoter region from the soybean *lbc3* gene (Christensen *et al.*, 1989). This promoter directs nodule-specific expression of reporter genes in different heterologous legume systems such as *Lotus corniculatus* (Stougaard *et al.*, 1986) and alfalfa (de Brujin *et al.*, 1989). Alfalfa was genetically transformed by co-cultivation with *Agrobacterium tumefaciens* harboring pMRGA. Thirty-seven alfalfa plants were regenerated on selective media from 60 co-cultivated explants. The presence of the transgenes in regenerated plants was initially confirmed by PCR of both, the *nptII* marker gene and the junction of *lbc3* promoter and *NADH-GOGAT* gene. This analysis showed that 11 independent transformants contained both transgenes, and were designated as GA (*GOGAT* antisense) plants. Also, transgenic alfalfa plants containing only the marker gene from pBin19 were obtained, and used as control plants.

The 11 GA plants obtained and one control plant were propagated clonally as stem cuttings which when rooted were inoculated with *S. meliloti* strain 2011. Small clonal populations of each GA and control line were assayed for nodule NADH-GOGAT activity. Four of the 11 transgenic lines had consistently reduced nodule NADH-GOGAT activity, as compared to the control. These four primary

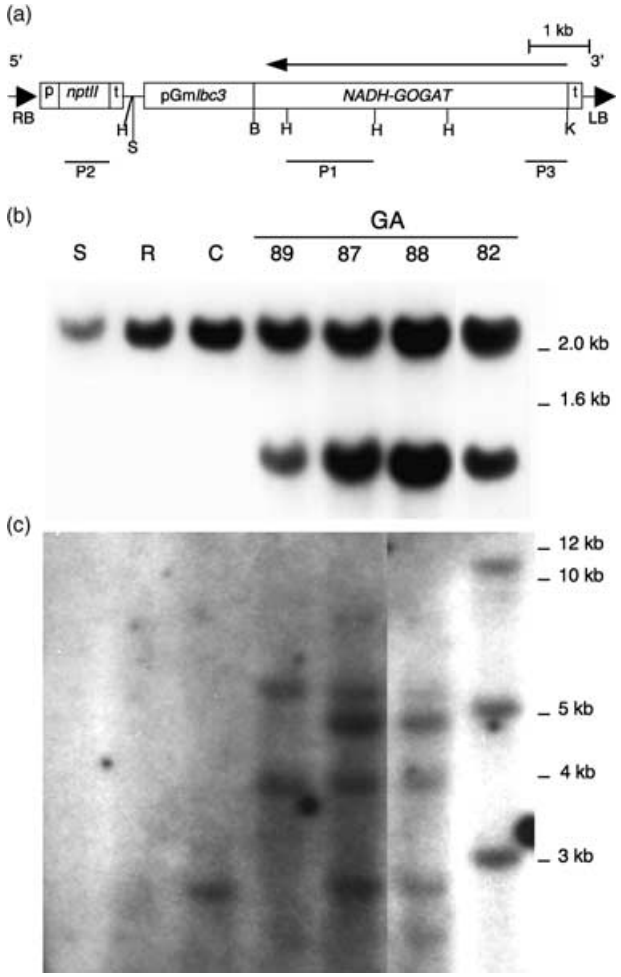

Figure 1. Schematic representation of the T-DNA region of plasmid pMRGA and DNA blot analysis.
(a) Plasmid pMRGA harbors the marker gene *nptII* fused to the nopaline synthetase promoter (p) and nopaline synthetase terminator (t). A partial DNA fragment of 5.3 kb from the alfalfa *NADH-GOGAT* cDNA is fused in antisense direction with respect to the Gm*lbc3* promoter and t. Both genes are flanked by the right (RB) and left (LB) borders of the *A. tumefaciens* T-DNA. P1–P3 indicated fragments used as probes for DNA- (P1 and P2) and RNA-blot (P3) analyses. B: *Bam*HI; H: *Hin*dIII; K: *Kpn*I; and S: *Sac*I.
(b and c) Southern blot analyses of transgenic GA and control plants. DNA (10 μg) was digested with *Hin*dIII and probed with P1 (b) or with a PCR-amplified fragment from *nptII* gene (c). Molecular weight markers are shown. S: Saranac; R: RegenSY27x; C: control line; and GA: *NADH-GOGAT* antisense lines.

transformants, designated GA89, GA87, GA88, and GA82 were selected for further study. For maintenance, these lines were grown in non-symbiotic conditions, watered with nutrient solution containing nitrate as nitrogen source. Under non-symbiotic conditions the growth of GA plants was similar to wild-type plants.

The stable integration of the *NADH-GOGAT* transgene in the four GA primary transformants, was confirmed by DNA blot analysis (Figure 1b). Using the P1 fragment (Figure 1a) as probe, the expected hybridization bands corresponding to internal fragments from the antisense transgene and from the genomic *NADH-GOGAT* sequence were observed in the *Hin*dIII-digested total DNA (Figure 1b). The hybridization pattern of the four GA plants showed a band of approximately 1.5 kb that corresponded to the internal fragment of cDNA used as probe, and a band of approximately 2 kb that corresponded to the genomic *NADH-GOGAT* sequence, nucleotide 8380–10443 which includes exons 14 and 15 plus introns 13–15 flanked by part of exons 13 and 16 (Vance *et al.*, 1995) (Figure 1b). The hybridization pattern of the alfalfa negative control plants analyzed: one non-transformed plant from the Saranac cultivar used for sequencing the *NADH-GOGAT* gene (Gregerson *et al.*, 1993; Vance *et al.*, 1995); one non-transformed plant from the RegenS cultivar used for transformation; and one control plant, showed only the expected larger band corresponding to the alfalfa *NADH-GOGAT* genomic fragment (Figure 1b).

To assess copy number of inserted transgenes, total DNA digested with *Hin*dIII was probed with the P2 *nptII* gene fragment (Figure 1a). The two non-transformed alfalfa plants lacked a hybridization band, while the control plant showed only one copy of the transgene (Figure 1c). Data from Figure 1(c) indicated that multiple copies of the transgenes were present in each transgenic line: GA89 line showed at least two copies, GA87 and GA88 showed at least four copies each, and GA82 showed at least three copies (Figure 1c). However, these data (Figure 1c) may not reflect the presence of the entire *NADH-GOGAT* antisense transgene. The different hybridization patterns observed indicate that each GA plant arose from independent transformation events.

Antisense inhibition of NADH-GOGAT *gene expression in transgenic alfalfa GA lines*

The effect of the antisense *NADH-GOGAT* construct on homologous *NADH-GOGAT* gene in alfalfa nodules was determined at the level of transcripts, protein, and enzyme-specific activity. Molecular analysis was performed 21 days post-inoculation (dpi) with *S. meliloti*, when mature alfalfa nodules showed the highest activity of ammonium assimilating enzymes (Egli *et al.*, 1989).

Steady-state levels of *NADH-GOGAT* transcripts in nodules of GA alfalfa lines were determined by RNA blot analysis using total RNA extracted from nodules of GA and control lines (Figure 2a) probed with P3 internal fragment of *NADH-GOGAT* cDNA (Figure 1a). All the GA lines analyzed showed a significant reduction in the abundance of *NADH-GOGAT* transcript as compared to the control line (Figure 2a). Densitometric quantification of at least three blots indicated that the reduction was higher in the GA89 line (62% ± 14) than in the GA87 (35% ± 5), GA88 (50% ± 11), and GA82 (48% ± 12) lines.

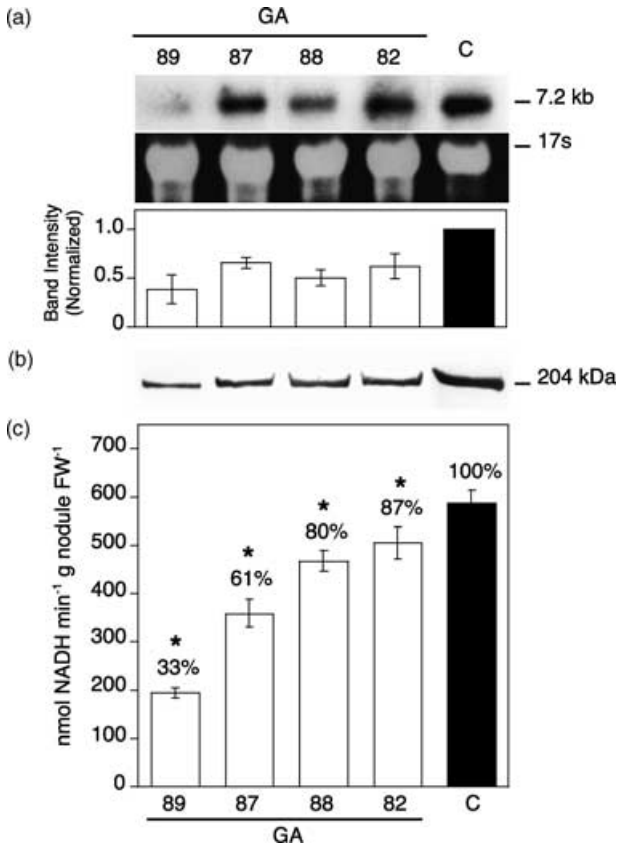

Figure 2. Analysis of *NADH-GOGAT* gene expression in transgenic antisense lines nodules at 21 dpi.
(a) RNA gel blot analysis of *NADH-GOGAT* mRNA. Total nodule RNA (20 μg) from transgenic and control lines was hybridized with P3 (shown in Figure 1a) and EtBr-stained 17S rRNA functioned as loading control. The relative intensity of the bands hybridizing with P3 was quantified using the NIH IMAGE version 1.61 program. Values are mean ± SE of at least three measurements.
(b) Western blot analysis. Protein extracts (50 μg) were probed with NADH-GOGAT antibody. Shown is a representative immunoblot out of a total of three experiments.
(c) Nodule-specific activity of NADH-GOGAT. Values are mean ± SE of at least 10 measurements. ★Significant difference ($P = 0.05$).

The expression of alfalfa NADH-GOGAT polypeptide was determined by immunoblot analysis using specific alfalfa NADH-GOGAT polyclonal antibodies (Anderson *et al.*, 1989). The data from Figure 2(b), showed that in all GA lines NADH-GOGAT protein was reduced as compared to the control line. Quantification of at least three protein blots by image densitometry, demonstrated a consistent reduction in NADH-GOGAT protein in the GA89, GA87, GA88, and GA82 of 69% (±6), 32% (±14), 22% (±11), and 11% (±4), respectively, from the control.

The data of nodule NADH-GOGAT-specific activity, averaged from different experiments and the percentage of remaining activity with respect to the control are shown in Figure 2(c). The greatest reduction in NADH-GOGAT-specific activity was observed in GA89 line, in agreement with the reduction in its RNA and protein levels.

Our data on the activity of NADH-GOGAT at different stages of alfalfa nodule development were in agreement with those reported by Egli *et al.* (1989): it was maximal at around 19 dpi, and then decreased to very low levels at later stages of the symbiosis with *S. meliloti*. At 35 dpi, the percentage of remaining NADH-GOGAT-specific activity in the nodules of each of the four GA lines (data not shown) was similar to the remaining activity described for 21 dpi (Figure 2c). Whereas a drastic drop of the activity was observed at 84 dpi in the four GA lines compared to the control, the remaining activity ranged between 40 and 50% (data not shown).

Analysis of the progeny (T_1) from GA primary transformants

Seeds from each GA line were obtained after crossing two different individuals from the same line (essentially self-crosses but harboring the transgenes); these were germinated, grown and inoculated with *S. meliloti.* The progeny T_1 plants analyzed by DNA-blot: eight plants from GA89; eight plants from GA87; and eight plants from GA88 inherited the trasgene (data not shown). Only one T_1 plant from GA87 did not inherit the transgene and had a normal phenotype.

A physiological characterization of T_1 plants derived from the primary transformants of lines GA89, GA87, and GA88 was undertaken in order to assess whether the *NADH-GOGAT* antisense phenotype correlated with the presence of the inherited transgene. Nodule NADH-GOGAT-specific activity from eight GA89 progeny plants showed a reduction ranging from 88 to 60% that of the control line. Three GA89 T_1 plants were further characterized as shown in Figure 3. These had reduced *NADH-GOGAT* transcript levels of 90–50% (Figure 3a) accompanied by reduced protein levels of 85–70% (Figure 3b) and a reduction in enzyme activity of *c.* 85% (Figure 3c). Of the five GA87 T_1 plants analyzed, one did not show an antisense phenotype, while the rest showed a reduction of NADH-GOGAT activity ranging from 60 to 50%. Further characterization of the T_1 GA87 plant showed a reduction of *NADH-GOGAT* RNA of 57% (Figure 3a) and protein levels of 30% (Figure 3b) that correlated with a 50% reduction in enzyme activity. Eight T_1 GA88 plants showed a reduction in nodule NADH-GOGAT activity of 50–20%. One T_1 GA88 plant analyzed also had reductions of 58% in *NADH-GOGAT* RNA level (Figure 3a), 24% in the protein level (Figure 3b), and 54% in enzyme activity (Figure 3c).

Nitrogen and carbon assimilation in nodules of GA antisense lines

Previous studies have demonstrated that key symbiotic enzymes for primary nitrogen assimilation, such as GS,

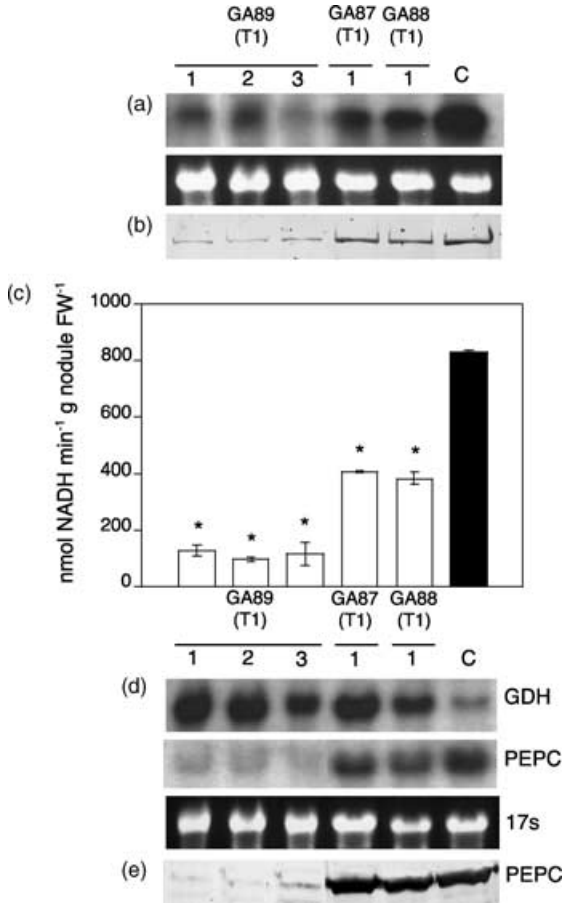

Figure 3. Analysis of NADH-GOGAT and related root nodule enzyme activity in progeny (T_1) plants from GA primary transformants. Data from three T_1 plants from GA89, one from GA87 and one from GA88 as compared to a T_1 control plant are shown. (a–c) NADH-GOGAT expression, performed as described in Figure 2.
(a) RNA gel blot analysis and EtBr-stained 17S rRNA as loading control.
(b) Western blot analysis.
(c) Specific enzyme activity. ★Significant difference ($P = 0.05$).
(d, e) Symbiotic enzyme expression. (d) RNA gel blot analysis performed as in Figure 3, probed for *NADH-GDH* and *PEPC*, and EtBr-stained 17S RNA.
(e) Western blot analysis for PEPC performed as in Figure 5.

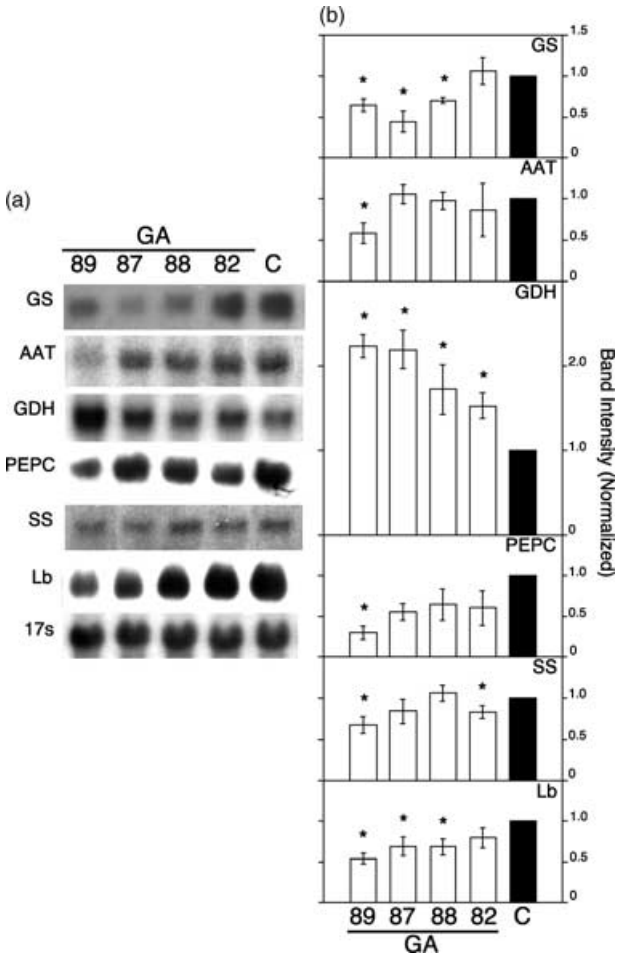

Figure 4. RNA gel blot analysis of key symbiotic enzymes.
(a) Total RNA (20 μg) isolated from 21-dpi nodules of GA and control lines was probed for the presence of transcripts encoding for: glutamine synthetase (GS), aspartate aminotransferase (AAT), NADH-glutamate dehydrogenase (GDH), phosphoenolpyruvate carboxylase (PEPC), sucrose synthase (SS), and leghemoglobin (Lb). Probed 17S rRNA as loading control.
(b) Quantification of intensity of the bands hybridizing with each probe using the NIH IMAGE version 1.61 program. Values are mean ± SE of at least three measurements. ★Significant difference ($P = 0.05$).

NADH-GOGAT, AAT, PEPC, and SS are induced in nodules during effective symbioses (Fedorova *et al.*, 1999; Vance *et al.*, 1994). These enzymes are postulated to have a common regulatory mechanism, and so an alteration in nodule *NADH-GOGAT* gene expression could potentially affect the expression of the other genes. To test this hypothesis, nodule RNA and protein levels as well as the enzyme activities of GS, AAT, PEPC, and SS were determined at 21 dpi in the four GA and the control lines (Figures 4 and 5). The RNA, protein levels, and enzyme activities of nodules from the GA82 line were not significantly different from the control (Figures 4 and 5). The transcript abundance of *GS* was significantly reduced in the GA89, GA87, and GA88 lines by 35% (±7), 56% (±13), and 30% (±4), respectively (Figure 4). However, the specific activity and protein level of nodule GS did not vary among any of the plants studied (data not shown). The amount of *AAT* RNA (42% ± 12) (Figure 4), protein (23% ± 2) and activity (28% ± 8) were significantly reduced in nodules of GA89 plants (Figure 5a). The transcript abundance of *PEPC* was significantly reduced by 70% (±8) in nodules of GA89 (Figure 4) and correlated with low levels of protein and enzyme activity (Figure 5b). By comparison, the protein level of PEPC in GA89 showed a greater reduction of 84% (±1), than of enzyme activity (69% ± 2) (Figure 5b). This is consistent with previous studies showing selective degradation of PEPC in ineffective alfalfa nodules (Pladys and Vance, 1993). PEPC activity was also significantly reduced

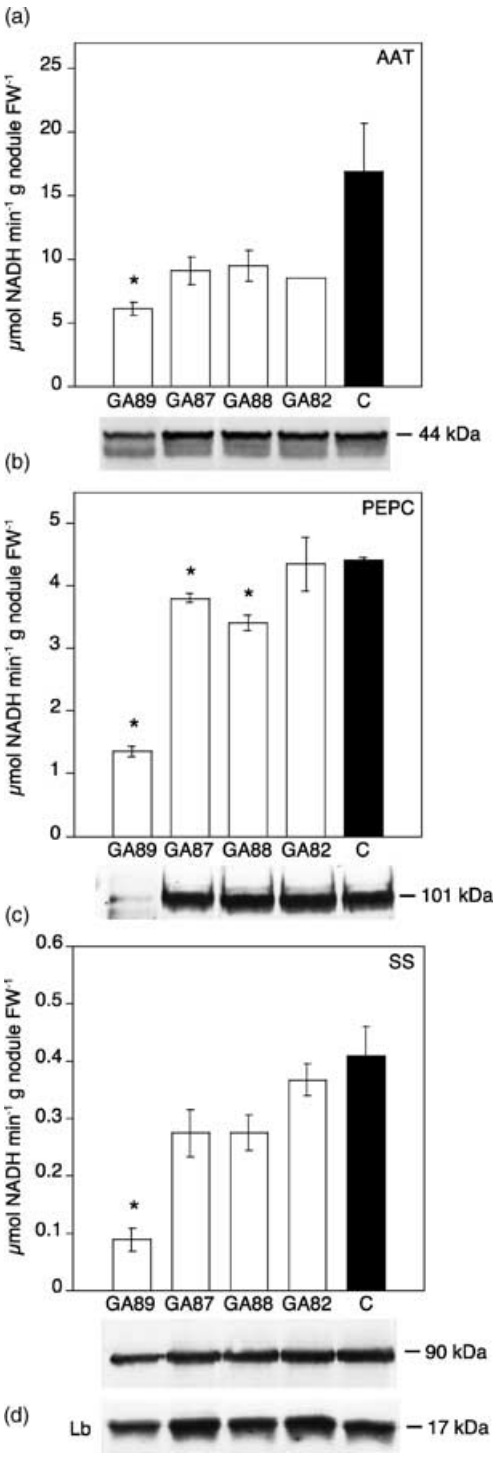

Figure 5. Specific activities and protein levels of key symbiotic enzymes in alfalfa nodules at 21 dpi.

Enzyme activities and immunoblot analysis for AAT (a), PEPC (b) and SS (c), and immunoblot analysis for Lb (d) were performed in 21-dpi nodule extracts from GA (white bars) and control (C) lines (dark bars). For immunoblot analysis, variable amounts of total soluble protein were separated in polyacrylamide gels containing SDS at different concentrations (as indicated in Experimental procedures). The molecular mass (kDa) corresponding to each polypeptide is indicated. ★Significant difference ($P = 0.05$).

in nodules of GA87 and GA88 (Figure 5b). The transcript abundance of *SS* showed a significant reduction of 33% (±10) (Figure 4) that correlated with the reduction in protein level (31% ± 5) in GA89 nodules (Figure 5c). However, a greater reduction of 78% (±5) in enzyme activity was observed (Figure 5c).

Although the role of NADH-dependent glutamate dehydrogenase (NADH-GDH) in plants has not been precisely defined as yet, it has been described as a catabolic rather than a biosynthetic enzyme (Lam *et al.*, 1996). The transcripts for *NADH-GDH* were increased, *c.* twofold, in the nodules of the four GA lines analyzed as compared to control nodules (Figure 4). The transcripts for Fd-GOGAT were not detected in nodules of any of the alfalfa plants analyzed (data not shown).

Leghemoglobin (Lb) has a crucial role in symbiosis by facilitating oxygen diffusion to actively respiring bacteroids (Appleby, 1984). Leghemoglobin RNA showed a reduction in nodules of GA89 (46% ± 7), GA87 (31% ± 12) and GA88 (32% ± 10) plants (Figure 4) and Lb protein abundance showed a reduction in nodules of GA89 (18% ± 7) (Figure 5d).

The T_1 plants analyzed showed the expected alterations in the expression of genes coding for C/N assimilation enzymes (Figure 3). *NADH-GDH* transcript abundance appeared to increase in all the T_1 plants analyzed, as compared to the control (Figure 3d). The three GA89 T_1 plants showed a reduction in *PEPC* transcript level that corresponded to a reduction in protein level (Figure 3d,e).

Amino acids synthesized in alfalfa nodules from carbon and nitrogen assimilation favors the synthesis of amides, such as asparagine and glutamine, which are preferentially transported via xylem to other plant organs (Temple *et al.*, 1998b; Vance *et al.*, 1994). The HPLC analysis of amino acids content showed that glutamate, glutamine, and alanine were the most abundant amino acids detected in all the nodules analyzed. No significant difference was observed among the amino acids content in nodules from control versus GA88 and GA82 plants (data not shown). In contrast, in nodules of GA89 plants, the concentration of the most abundant amino acids was decreased by some 70%, while the concentration of other amino acids present in smaller amounts was reduced by 50% (Table 1). In nodules of GA87 plants, the concentration of glutamate (30%), glutamine (20%) and alanine (50%) were significantly reduced with respect to nodules of control plants (Table 1). A TLC analysis of amino acids content in the nodules of GA and control plants showed that asparagine was one of the most abundant amino acids. This analysis showed that asparagine was reduced around 67 and 41% in nodules of GA89 and GA87 plants, respectively (data not shown). However, the HPLC method that we used (Table 1) did not allow us to quantify the asparagine concentration in nodules because it is hydrolyzed in the acidic environment

Table 1 Metabolite content in transgenic GA lines nodules at 21 dpi

	Amino acids (μmol g nodule FW^{-1})				Carbohydrates (mg g nodule FW^{-1})		
LINE	Glu	Gln	Ala	Σ	Glc	Fru	Sac
GA89	0.87 (±0.14)*	1.37 (±0.32)*	0.28 (±0.06)*	0.85 (±0.08)*	0.96 (±0.05)*	0.68 (±0.05)	1.92 (±0.14)*
GA87	1.75 (±0.13)*	3.73 (±0.28)*	0.66 (±0.11)*	1.15 (±0.07)	0.97 (±0.06)*	0.97 (±0.07)	2.50 (±0.15)*
C	2.53 (±0.26)	4.73 (±0.33)	1.26 (±0.23)	1.55 (±0.18)	0.76 (±0.06)	0.73 (±0.12)	1.43 (±0.16)

Values are the mean (±SE) from at least three measurements.
Σ, sum of the concentration of arg, asp, gly, ile, iso, lys, met, phe, ser, thr, and val.
*Significant difference versus control ($P = 0.05$).

used to prepare the samples. These data are in agreement with the diminished enzyme activities observed in nodules from GA89 and GA87 plants (Figure 5).

The inhibition of carbon metabolic enzymes such as PEPC and SS (Figure 5) and the impairment in ammonium assimilation and amino acids synthesis (Table 1) in the nodules of GA plants could potentially affect the concentration of carbon compounds which are the substrates or products of those enzymes. To test this hypothesis, we measured the concentration of sugar and organic acids in the nodules of the antisense and control plants (Table 1). The concentrations of all carbon metabolites determined showed no significant differences in nodules of GA88 and GA82 plants as compared to the controls. However, carbohydrate determinations revealed that sucrose content was increased some 35 and 75% in the nodules of the GA89 and GA87 plants, respectively. Fructose concentration did not vary significantly, while glucose concentration increased about 25% in both GA plants. This is consistent with the observed inhibition of nodule SS activity (Figure 5c). The concentration of nodule organic acids citrate, malate and succinate did not vary in the GA plants with respect to control. However, oxalate content was increased some twofold in the nodules of the GA89, GA87, and GA88 plants with respect to control plants (data not shown). The accumulation of oxalate may be related to an adaptive response to detoxify ammonia through the formate pathway.

Characterization of the symbiotic phenotype of GA antisense lines

Plants from GA89 and GA87 antisense lines formed more nodules than control plants (Figure 6a). We interpreted this as a compensatory response of the plant to the diminution of ammonium assimilation. However, the total nodule dry weight of plants from GA89 and GA87 antisense lines was significantly reduced by 35% (±4) and 29% (±6), respectively, from control plants (Figure 6b). Accordingly, nodules from the GA89 and GA87 lines showed morphological alterations. Not only was the average dry weight of individual nodules reduced (data not shown) but nodules were also smaller and yellowish as compared to wild-type nodules. The morphology of nodules from the GA89 was severely altered in comparison to wild-type nodules (Figure 6c,d). The smaller size and a yellowish color were evident in longitudinal sections of nodules from GA89 plants (Figure 6e). Nodule sections from at least three different nodules from each plant (20 observations in total) were evaluated. The histological analysis of control and GA89 nodules (Figure 6f,g) showed the typical developmental zones reported by Vasse *et al.* (1990): the meristematic zone (I), infection zone (II), the amyloplastic-rich interzone between II and III zones (*), the nitrogen-fixing zone (III) and the senescent zone (IV). As compared to control nodules, in nodules from GA89 plants the zones I–III were smaller while the senescent zone (IV) was increased in size (Figure 6f,g). Broad differences were observed in the nitrogen fixation zone (zone III), there were fewer infected cells in GA89 nodules versus control nodules (Figure 6h,i). Moreover, infected cells of GA89 nodules were smaller than those seen in nodules of the control (Figure 6h,i). In addition, early senescence can be observed in GA89 nodules, as indicated by agglutination of the bacteroids in infected cells (Figure 6i).

The altered morphology of the nodules from the GA89 (Figure 6) and GA87 (data not shown) plants, represented by a reduced nitrogen fixation zone accompanied by an increased senescent zone, reflects the reduction in symbiotic nitrogen fixation. A significant decrease (67% ± 3 and 58% ± 7) in nitrogenase-specific activity was detected in nodulated GA89 and GA87 plants, respectively, with respect to control plants (Figure 7a). Lower symbiotic nitrogen fixation in GA89 and GA87 plants was accompanied by the significant decrease in the foliage fresh weight (41% ± 4 and 29% ± 6) (Figure 7b). In addition, the total nitrogen content of the foliage of GA89 plants was decreased (38% ± 6) at 21 dpi (Figure 7c), and a diminution of foliage nitrogen content (37% ± 2) in GA87 plants was evident at later stages of the symbiosis.

The inoculated GA89 and GA87 plants showed the chlorotic symptoms resulting from the lack of sufficient nitrogen in aerial organs. While the chlorophyll content of the leaves of control plants was 37 (±1) nmol cm^{-2}, the leaves from GA89 line showed 28 (±2) nmol cm^{-2}

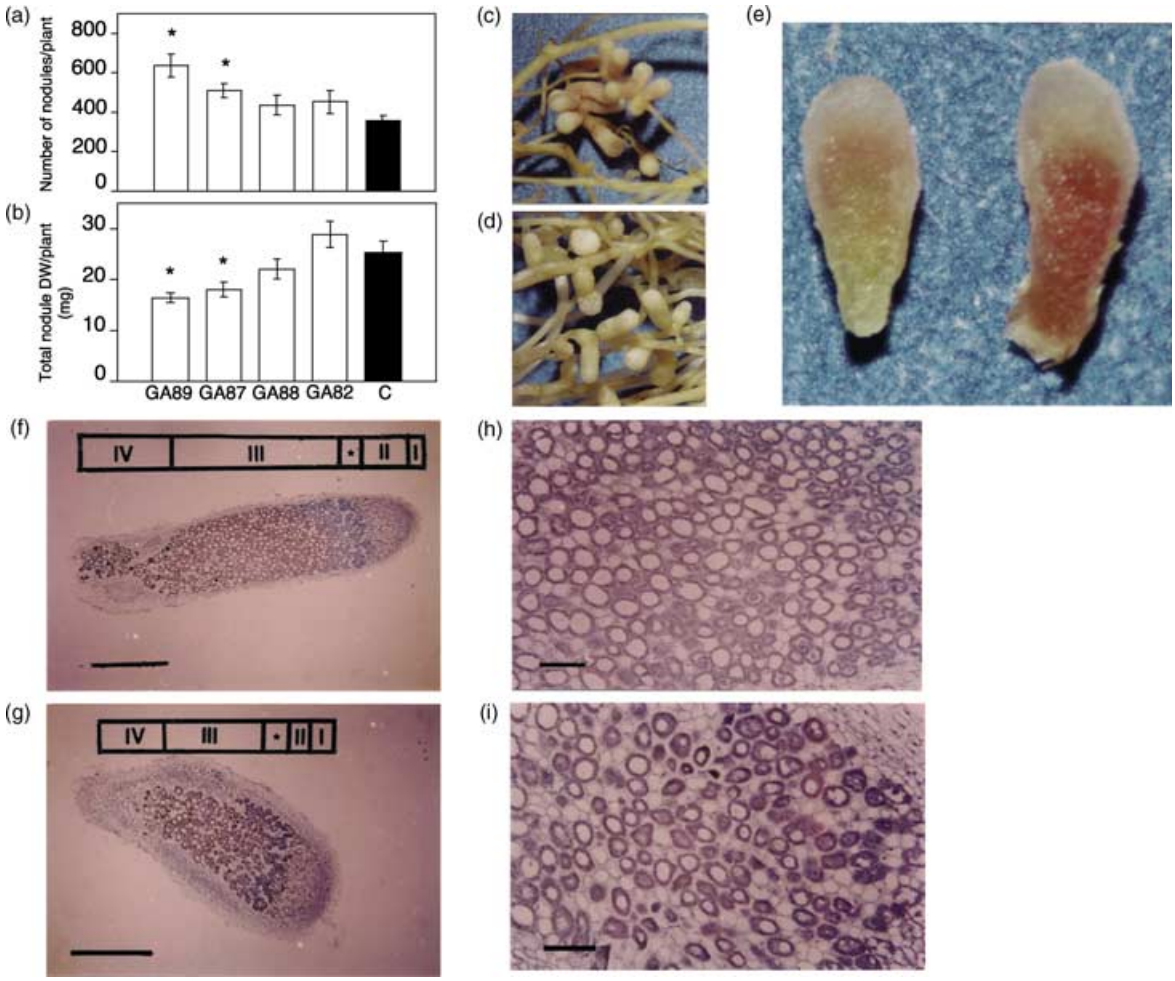

Figure 6. Nodulation phenotype of GA lines.
Number of nodules (a) and nodule dry weight (b) of GA (white bars) and control (C) (dark bar) lines at 21 dpi. ★Significant difference ($P = 0.05$). Nodules from control (c) and GA89 (d) lines. (e) Longitudinal sections of a representative nodule from GA89 (left) and C (right) lines. (f–i) Bright-field photographs of longitudinal sections of control (f) and GA89 (g) lines. Nodule zones as designated by Vasse *et al.* (1990) are indicated. Bar = 500 μm. (h, i). Magnification of the nitrogen fixation zone (III) from (f) and (g), respectively. Bar = 100 μm.

and those from GA87 line showed 32 (±1) nmol cm^{-2}. The leaf area of inoculated GA89 and GA87 plants was reduced by 30% (±3) and 15% (±5), respectively, as compared to control plants. The GA89 and GA87 plants had reduced light-saturated photosynthesis by around 40–30%, respectively, from control plants. Figure 7(d) shows representative 21-dpi alfalfa plants from the GA89 and control lines, the reduced growth of the antisense plant was evident.

Discussion

Here, we extend the understanding of root nodule NADH-GOGAT by showing that specific reduction of nodule NADH-GOGAT activity results in impaired nitrogen assimilation, reduced plant N and growth, early senescence of nodules, and reduced symbiotic nitrogen fixation. The reduction in nitrogen assimilation and fixation was accompanied by significantly increased nodule carbon in the form of sucrose, implying that carbon metabolism/flux was also modified by reductions in NADH-GOGAT activity. A previous study by Schoenbeck *et al.* (2000) on the characterization of a *NADH-GOGAT* antisense alfalfa plant reported that a reduction of nodule NADH-GOGAT enzyme activity by 40–50% resulted in only minor symbiotic alterations. In our study, a similar antisense technology approach was used, but the expression of antisense *NADH-GOGAT* cDNA fragment used was under the control of the nodule-specific Gm*lbc3* soybean gene promoter (Christensen *et al.*, 1989). Several *cis*-acting elements have been identified in the Gm*lbc3* promoter that are involved in nodule-specific expression (Ramlov *et al.*, 1993). We achieved a 70% reduction of nodule NADH-GOGAT using the Gm*lbc3* promoter and a concomitant alteration in symbiotic phenotype.

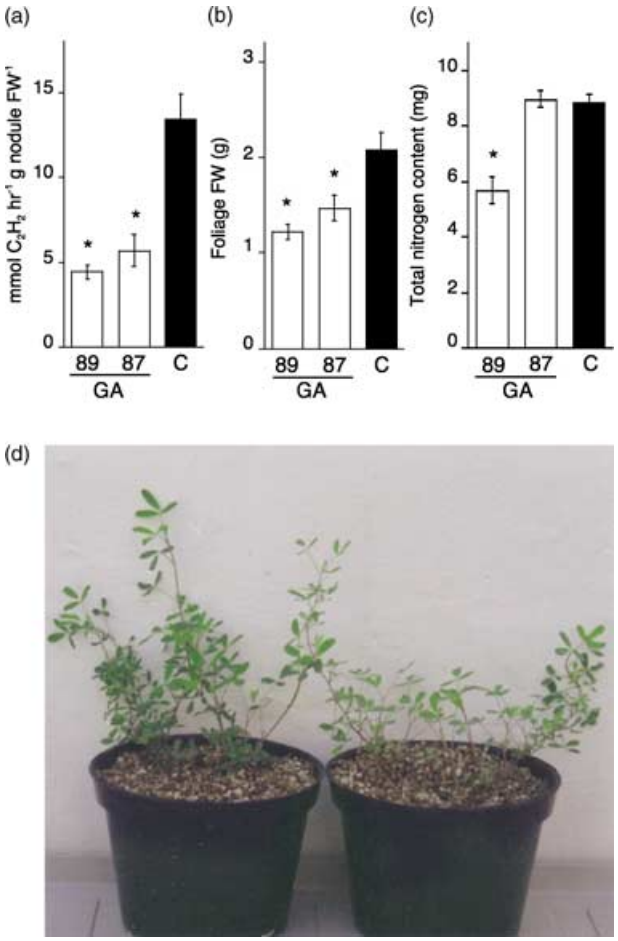

Figure 7. Symbiotic phenotype of GA89 and GA87 lines.
Nitrogenase activity (a), foliage fresh weight (b) and nitrogen content in foliage (c) in GA89, GA87 (white bars), and control (dark bars) lines at 21 dpi. Values are mean ± SE of at least five measurements. ★Significant difference ($P = 0.05$). (d) Foliage aspect of control (left) and GA89 (right) inoculated plants at 21 dpi.

Although the expected 5.3-kb anti-RNA molecule was not detected in nodules of GA plants, using double-stranded DNA probes, the mRNA concentration of *NADH-GOGAT* was decreased. These results suggest that the inhibitory effect of antisense RNA was exerted through the rapid degradation of double-stranded RNA species formed by complementary base pairing between *NADH-GOGAT* mRNA and antisense RNA (Sijen and Kooter, 2000).

The inhibition of *NADH-GOGAT* gene expression in alfalfa nodules resulted in a negative symbiotic effect, with the severity of the effect increasing in proportion to the inhibition of NADH-GOGAT enzyme activity in each of the four antisense transformants analyzed. The negative symbiotic effects occurred with concomitant reductions in the level of *NADH-GOGAT* transcript in the progeny of the GA lines obtained. All the T_1 plants analyzed had either similar or increased inhibition of *NADH-GOGAT* gene expression as compared to their parental line and showed similar negative pleitropic effects.

The affected symbiotic phenotype of GA89 and GA87 plants, which had greatest NADH-GOGAT inhibition *c.* 70 and 40%, respectively, strongly supports the concept that NADH-GOGAT activity limits the assimilation of ammonia from fixed nitrogen in alfalfa nodules. This work provides direct evidence for the role of NADH-GOGAT playing a critical role in nodule ammonia assimilation as proposed by Temple *et al.* (1998b) and Trepp *et al.* (1999a). Interestingly, antisense GS has little effect on symbiotic parameters (Temple *et al.*, 1998a).

In agreement with previous studies on the expression of key symbiotic enzymes during effective alfalfa symbiosis (Fedorova *et al.*, 1999; Vance *et al.*, 1994), most of the antisense *NADH-GOGAT* plants analyzed showed an accompanying inhibition in gene expression of other genes coding for C/N assimilation enzymes such as GS, AAT, PEPC and SS, and increased transcript abundance of *NADH-GDH*. It has been proposed that the signaling networks for C/N sensing enables plants to regulate metabolism and development in response to their internal C:N ratio and that the sensing and signaling systems are subjected to a 'matrix effect' in which certain functions and interactions occur depending upon species, cell types and developmental stages (Coruzzi and Zhou, 2001). Initial post-genomic studies in *Arabidopsis* are revealing the complexity of such network responses. The *Arabidopsis* microarray and RNA gel blot analyses reported by Wang *et al.* (2000) show diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. The impaired assimilation of fixed nitrogen of the GA89 and GA87 plants led to the accumulation of carbon compounds such as sucrose, glucose, and oxalate and to the decrease in N compounds such as amino acids and amides that translated into decreased shoot nitrogen. Thus, the sensing/signaling of the altered C:N ratios in the nodules of the antisense plants may trigger a complex response of the regulatory network that results in the altered transcription of several genes as we observed. Future post-genomic studies in model legumes such as *Medicago truncatula*, using mutant plants such as one lacking NADH-GOGAT, will enable us to more fully understand the complete regulatory networks or 'matrix effects' resulting from the C/N sensing or signaling in the nodules.

In addition to the concerted reduction in transcript levels of C/N assimilation genes, our results suggest that some of the symbiotic enzymes are also regulated at the post-transcriptional/transcriptional level. Although lower RNA level of *GS* was detected, the GA plants studied did not show reduction in protein concentration or enzyme activity. This is in agreement with previous reports that indicate that GS is controlled at the translational and post-translational levels in GS antisense alfalfa plants (Temple *et al.*,

1998a). We found that nodule PEPC activity is reduced in the GA87 and GA88 lines that did not show a reduction in transcript levels of this enzyme. It is known that nodule PEPC is regulated by phosphorylaytion/dephosphorylation cycle possibly mediated by changes in PEPC kinase activity and/or perturbation of substrate ATP levels in the cytosol and modulated by shoot photosynthate (Zhang and Chollet, 1997). The accumulation of sugars in nodules of the antisense plants having reduced ammonium assimilation may contribute a feedback inhibition of carbon metabolic enzymes such as SS.

The role of NADH-GDH in plant metabolism is still unresolved (Lam *et al.*, 1996). In alfalfa, NADH-GDH is expressed at low levels in the different zones of the nodules (Trepp *et al.*, 1999b). The presence of NADH-GDH enzyme activity in alfalfa nodules, did not correlate with nitrogen fixation since it was detected in ineffective nodules as well as in senescent nodules (Egli *et al.*, 1989). Our results on the increase in *NADH-GDH* transcript levels found in the early senescent nodules of GA plants, together with the results reported previously (Egli *et al.*, 1989; Trepp *et al.*, 1999b), indicate that the expression of NADH-GDH may be related to the recycling of ammonia during nodule senescence.

It has been reported that soybean bacteroids (Waters *et al.*, 1998) as well as pea bacteroids (Allaway *et al.*, 2000) may excrete some fixed nitrogen as alanine instead of or in addition to ammonia, to the plant nodule cells. To our knowledge, this phenomenon has not been reported for alfalfa bacteroids. We found that nodules from GA89 and GA87 lines had a reduction of alanine content of 80 and 50%, respectively, with respect to control nodules, and this does not seem to compensate the lack of glutamate and other derived nitrogen compounds needed for optimal plant growth.

Although nitrogenase data as measured by ARA must be interpreted with caution, we detected a decrease in nitrogenase activity on both nodule dry weight basis and the total activity per plant (data not shown) in the nodules of GA89 (70%) and GA87 (60%) than in the control. Nitrogenase activity in legume nodules is downregulated when nodulated plants are subjected to different treatments that affect plant C/N metabolism such as de-foliation, addition of nitrate or drought (Arrese-Igor *et al.*, 1997; Gordon *et al.*, 1997). Neo and Layzell (1997) proposed that the inhibition of nitrogenase operates via an increase in oxygen-diffusion resistance that affects the flux of oxygen into the bacteroids. In alfalfa nodules, oxygen concentration decreases abruptly from the apical region (zone I) to the interzone II–III, that precedes the nitrogen fixation zone III (Soupene *et al.*, 1995). We have shown that the nodules of the GA89 plants have an altered structure, with smaller zones II and III and increased senescent zone IV. Also, a reduction in Lb was observed in the nodules of GA plants that had yellowish color and less Lb transcript and protein. These alterations could affect the oxygen flux in the nodules of GA89 plants, and consequently the nitrogenase activity. We observed that the plant nitrogen deprivation, in symbiotic conditions, was accompanied by leaf chlorosis and impaired photosynthesis. The reduction in photosynthate supply may also affect nitrogen fixation in the nodules of GA89 plants.

In conclusion, the impaired symbiotic nitrogen fixation observed in GA89 and GA87 plants expressing antisense *NADH-GOGAT* appears to alter the metabolic flux of carbon and nitrogen compounds between photosynthetic and nodule tissues.

Experimental procedures

Vector construction

Plasmid pMRGA was constructed in three steps. In a first step, a 2-kb *Sal*I–*Bam*HI fragment (nucleotides −1956 to +46) containing the full-length promoter region of the soybean leghemoglobin *lbc3* gene (Christensen *et al.*, 1989) was introduced into *Sal*I–*Bam*HI-restricted pBI101 (Jefferson *et al.*, 1987) giving rise to plasmid pMR1. In the second step, a *Bam*HI–*Sac*I polylinker fragment derived from pSL1190 (Brosius, 1989) was introduced into *Bam*HI–*Sac*I-restricted pMR1, thereby replacing the *gus* gene and leading to pMR90. In the third step, the *Kpn*I–*Bam*HI fragment from pG7.2 (Gregerson *et al.*, 1993) containing 5.3 kb from the cDNA sequence of *NADH-GOGAT* from alfalfa nodules was cloned into the *Kpn*I–*Bam*HI sites of polylinker of pMR90 in antisense orientation with respect to the *lbc3* promoter generating plasmids pMRGA (Figure 1a).

Plant transformation and growth

Transgenic alfalfa (*M. sativa* L. cv. Regen SY27x) were generated essentially as described by Austin *et al.* (1995), using *A. tumefaciens* LBA4404 strain with the binary vector pMRGA or pBin19 (Bevan, 1984) as control. Well-developed embryos were recovered from callus cultured on media containing 50–100 μg ml^{-1} kanamycin. Rooted plantlets were transferred to pots with vermiculite and watered with B&D (Broughton and Dilworth, 1971) nutritive solution supplemented with 7.5 mM KNO_3 for maintenance in a glasshouse. The presence of the transgenes in regenerated plants was determined by PCR, using a pair of primers reported by Blake *et al.* (1991) for the amplification of a 785-bp fragment from the *nptII* gene and another pair of primers (5′-TCA CCC TCC TCC AAC AAG CCA-3′ and 5′-AAG ACG ATG AAG CCC AAG CAG-3′) for the amplification of a 470-bp fragment from the *lbc3* promoter–*NADH-GOGAT* gene junction. Plants testing positive for the transgenes were propagated clonally through stem cuttings that were rooted for around 5 weeks in pots with vermiculite and watered with nutrient solution. The propagated clones were then deprived of nitrogen for 2 weeks prior to inoculation with *S. meliloti* 2011 strain.

DNA isolation and gel blot analysis

Alfalfa genomic DNA was isolated from leaf tissue using the PUREGENE kit (GENTRA Systems, Minneapolis, MN). For Southern blot analysis, 10 μg of total DNA was digested with *Hin*dIII, separated by electrophoresis and transferred to Hybond-N^+ membranes (Amersham Life Sciences, UK). Hybridization was performed at 65°C using ^{32}P-labeled internal fragments from *NADH-GOGAT* cDNA (P1) or *nptII* (P2) gene (Figure 1a) as probes.

RNA isolation and gel blot analysis

Total RNA was isolated from 21-dpi nodules of transgenic alfalfa plants using TRIzol reagent (GibcoBRL Life Technologies Inc., Grand Island, NY) as described by the manufacturer. For Northern blot analysis, 20 μg of total RNA was separated in an agarose gel containing 1.5% formaldehyde and transferred by capillary blotting to Hybond N^+ membranes (Amersham Life Sciences, UK). Membranes were hybridized with ^{32}P-labeled probes at 65°C overnight. The P3 fragment from the *NADH-GOGAT* cDNA clone (Figure 1a), internal fragments from cDNA clones from bean nodule-induced *AAT*, *PEPC*, and *SS* transcripts (kindly provided by the group of Dr M. Lara, Nitrogen Fixation Research Center, UNAM, Mexico), as well as fragments from cDNA clones from alfalfa cytosolic *GS1*, *NADH-GDH, Fd-GOGAT*, and *Lb* were used as probes. The relative intensity of the bands hybridizing to the different probes, from at least three developed blots for each probe, was quantified using NIH IMAGE version 1.61 program. The data were analyzed by ANOVA.

Protein immunoblot analysis

Nodules were ground at 4°C in a mortar with the particular extraction buffer reported for each enzymatic assay (see below). Protein samples were separated by electrophoresis in SDS–PAGE gels. The total amount of protein and the percentage of acrylamide in the gel used for each enzyme was as follows: for NADH-GOGAT and PEPC, 50 μg protein and 7% gels; for GS, 40 μg protein and 10% gels; for AAT, 20 μg protein and 12.5% gels; for SS, 20 μg protein and 10% gels; and for Lb, 2 μg protein and 15% gels. After separation by electrophoresis, proteins were transferred to nitrocellulose membranes. The respective proteins were detected by incubating the blots first with specific anti-sera directed against alfalfa NADH-GOGAT (Anderson *et al.*, 1989), AAT (Griffith and Vance, 1989) PEPC (Miller *et al.*, 1987), Lb (Egli *et al.*, 1989), bean nodule GS1 (kindly provided by Dr M. Lara) or soybean SS (Gordon, 1992) (kindly provided by Dr A.J. Gordon, Institute of Grassland and Enviromental Research, UK) and subsequently with an alkaline phosphatase-conjugated secondary antibody. For quantification the blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (Zymed Laboratories Inc., San Francisco, CA), as described by manufacturer. The relative density of the developed blots, from at least three blots for each enzyme, was quantified using NIH IMAGE version 1.61 program. The data were analyzed by ANOVA.

Determination of enzyme activities

Proteins were extracted from excised nodules and the activities of nodule enzymes were measured as previously described for NADH-GOGAT (Groat and Vance, 1981), GS (Ferguson and Sims, 1971), AAT (Gordon and Kessler, 1990), PEPC (Deroche *et al.*, 1983), and SS (Morrell and Copeland, 1985). Protein was measured by the Bradford (1976) method. Nitrogenase activity was determined in detached, 21-dpi nodulated roots by the acetylene-reduction assay (ARA). After the assay, nodule dry weight was determined. The data were analyzed by ANOVA.

Metabolite determinations

Amino acids were extracted with 80% ethanol from 100 mg fresh harvested nodules (21 dpi). The extracts were incubated at 37°C for 2 h and centrifuged (12 000 r.p.m.), then the pellets were lyophilized. Amino acids were determined by HPLC reverse phase analysis using a pre-column derivation technique with 9-fluoromethyl chloroformate and a Nova-Pack C18 column (Waters International, Hertfordshire, UK). For carbohydrates determinations, harvested nodules were ground in cold 1 M $HClO_4$ (100 mg nodule FW ml^{-1}). The extracts were processed as described by Ferrario-Méry *et al.* (1997). Glucose, fructose and sucrose were determined by enzymatic reactions (1 ml final volume) coupled to NADH production as described by Gordon *et al.* (1999), with the following modifications. For glucose determinations, the reaction mixture contained 1 mM ATP, 1.5 mM NAD, 0.18 U ml^{-1} glucose-6-P dehydrogenase and 0.2 U ml^{-1} hexokinase. For fructose and sucrose determinations, 2 U ml^{-1} phospho-gluco-isomerase and 20 U ml^{-1} acid invertase were added, respectively. Organic acids were determined as described by Tesfaye *et al.* (2001). Total N content of dry shoot samples from 21-dpi plants was determined with an Antek 7000 (Antek Instruments Inc., Houston, TX), as reported by Cevallos *et al.* (1996).

Microscopy

Harvested nodules were rapidly fixed and processed as reported by Trepp *et al.* (1999a). The embedded tissues were sectioned (5 μm) and affixed to poly-L-Lys-coated slides. After xylene deparaffinization, the sections were stained with a double-stained (safranin–fast green), dehydrated and mounted with Permount (Fisher Scientific). Sections were viewed with Axioskop 2 (Zeiss) and photographed with a MC80 DX camera (Zeiss).

Chlorophyll content and photosynthesis

Chlorophyll was extracted from freshly harvested fully expanded leaves using 80% acetone, and quantified as reported (Graan and Ort, 1984). Leaf gas exchange (Li-6400, Li-Cor Inc., Lincoln, NE) was used to measure light-saturated net CO_2 assimilation rate (Nogués *et al.*, 1998).

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